MAMMALIAN LIPOATE ACETYLTRANSFERASE: MOLECULAR WEIGHT DETERMINATION BY GEL FILTRATION IN THE PRESENCE OF GUANIDINIUM CHLORIDE

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Received 6 February 1980

1. Introduction

Lipoate acetyltransferase (E2) forms the core of the pyruvate dehydrogenase multienzyme complex (reviewed [1,2]). The subunit M_r of mammalian E2 has been a matter of controversy. Using SDS–polyacrylamide gel electrophoresis M_r 74 000–76 000 has been found for the bovine as well as porcine kidney and heart enzyme [3–6]. The same value was reported for pig heart E2 in a study employing gel filtration and sedimentation equilibrium analysis in the presence of 6 M guanidine–HCl [4]. However, by this latter method, the subunit M_r of bovine kidney and heart E2 was determined to be ~52 000 [3].

Knowledge of the subunit M_r of E2 is indispensable to elucidate the molecular structure of the pyruvate dehydrogenase complex. In fact, due to the uncertainty about the E2 subunit, contradictory models have been proposed for the polypeptide chain stoicheiometry of the mammalian E2 core as well as the enzyme complex [3–5]. We therefore saw fit to redetermine the subunit M_r of bovine kidney E2 by gel filtration using Sephacryl S-300 as the gel matrix.

2. Experimental

2.1. *Materials*

Pyruvate dehydrogenase complex was purified from bovine kidney and E2 isolated from the complex as in [6]. Its papain fragment was obtained as in [7]. Sephacryl S-300 and blue dextran-2000 were from Pharmacia, Uppsala. Guanidine–HCl was from Merck, Darmstadt, or Sigma, St Louis, and was purified according to [8]. Cytochrome *c*, aldolase, lactate dehydrogenase, and phosphorylase *a* were from Boehringer, Mannheim, chymotrypsinogen and DNP-Ala from Serva, Heidelberg, and bovine serum albumin from Behringwerke, Marburg. Iodo[1-^{14}C]acetamide (57 Ci/mol) and [^{3}H]NSP (43 Ci/mmol) were from the Radiochemical Centre, Amersham.

2.2. *Methods*

The calibrating proteins as well as E2 were radiolabelled either with iodo[1-^{14}C]acetamide according to [8] or were treated first with [^{3}H]NSP at pH 8.5 as in [9] and then with unlabelled iodoacetamide in the presence of 6 M guanidine–HCl [8].

Gel filtration was performed according to [10] but using Sephacryl S-300 superfine instead of S-200 (gel bed dimensions, 1.6 × 83 cm). The elution buffer was 6 M guanidine–HCl (pH 5.0) (flowrate 4.8 ml/h). Fractions of ~0.88 ml (11 min) were collected. To improve reproducibility, the elution positions were determined by weight rather than by volume [11]. Blue dextran and DNP-Ala were used as the markers for the void and total volume, respectively, and were localized by measuring A_{630} and A_{360}. To determine the elution positions of the labelled proteins, samples of each fraction were counted for radioactivity, except for cytochrome *c* which was monitored at 410 nm. To obtain a calibration curve, K_d values were calculated according to [10] and plotted according to [12] or [13].

SDS–polyacrylamide gel electrophoresis was per-

Abbreviations: E2, lipoate acetyltransferase (EC 2.3.1.12); M_r, molecular weight; SDS, sodium dodecylsulfate; DNP-Ala, dinitrophenyl-alanine; [^{3}H]NSP, *N*-succinimidyl-[2,3-^{3}H]-propionate

formed as in [6]. Localization of radioactivity in the Coomassie blue-stained gels was accomplished according to [14].

3. Results

3.1. *Gel filtration*

In our experiments, Sephacryl S-300 turned out to be a very suitable gel matrix for M_r determination of proteins in the presence of 6 M guanidine–HCl. Good linearity was obtained in the range from at least 11 000–93 000 M_r when the data were plotted either according to [12] ($r^2 = 0.998$, fig.1) or [13] ($r^2 = 0.989$, not shown).

Bovine kidney E2 was eluted in a position corresponding to M_r 51 100 ± 1100 (or 53 600 ± 1200 when evaluated according to [13]). This value is in excellent agreement with the result of sedimentation equilibrium analysis [3] but disagrees with the app. M_r in SDS gel electrophoresis.

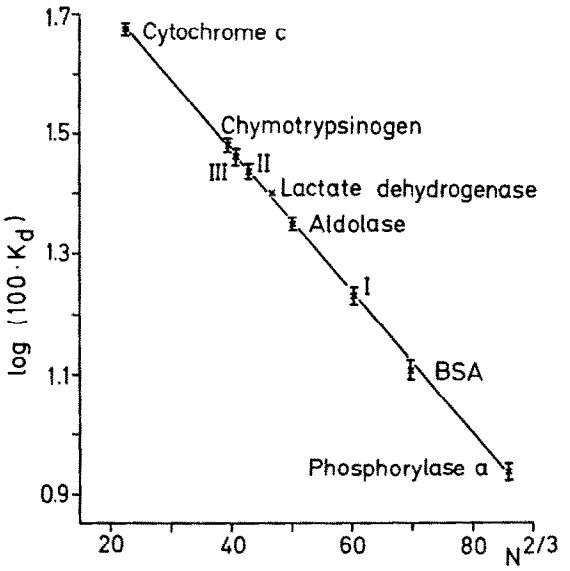

Fig.1. Plot of log 100 K_d vs $N^{2/3}$ [12] for the calibrating proteins as well as (I) E2, (II) the fragment formed from E2 during incubation with 6 M urea, and (III) the papain fragment of E2. N is the number of amino acid residues in the polypeptide chain. The following values were used for N (in parentheses, no. K_d determinations): phosphorylase *a*, 797 (3); bovine serum albumin (BSA), 579 (3); aldolase, 358 (3); lactate dehydrogenase, 315 (1); chymotrypsinogen, 245 (8); cytochrome *c*, 104 (12). K_d values found for the test proteins were: E2, 0.171 ± 0.004 (7); urea fragment, 0.271 ± 0.006 (5); papain fragment, 0.288 ± 0.008 (3).

The incorporation of radioactivity from [^{3}H]NSP into E2 was low (~3×10^7 cpm/mg, corresponding to ~0.05 mol/mol E2). We therefore tried to increase the amount of label by adding 6 M urea to the incubation mixture of E2 and [^{3}H]NSP. However, in this case, only part of the label was eluted at 52 000 M_r whereas ~50% of the radioactivity gave a peak corresponding to M_r 30 400 ± 900 (II in fig.1) which was quite insignificant (if at all detectable) in E2 samples labelled in the absence of urea. It appears that isolated E2 contains small amounts of a firmly bound protease which is activated in the presence of urea. The size of the fragment detected in these experiments is very similar to that of the principal stable fragment obtained from E2 after treatment with a lysosomal protease (M_r 29 200 ± 600 in SDS gel electrophoresis) [6].

We have reported [7] that after limited proteolysis of E2 with papain, the principal fragment detectable in polyacrylamide gels after staining with Coomassie blue has app. M_r ~26 000. When this fragment was analyzed by gel filtration, it was found to have M_r 28 000 ± 1200 (III in fig.1). This suggests that the papain fragment, unlike uncleaved E2, migrates normally in SDS gel electrophoresis and thus, may lack the region of the E2 chain responsible for its anomalous behaviour.

3.2. *SDS–polyacrylamide gel electrophoresis*

The following experiment was done to exclude the possibility that E2, when eluted from the column in a position corresponding to M_r ~52 000 was itself degraded proteolytically. The labelled material of the E2 peak was pooled, dialyzed extensively against water to remove the guanidine–HCl, freeze-dried and dissolved in the sample buffer for SDS gel electrophoresis. Unlabelled pyruvate dehydrogenase complex was added as a carrier and molecular weight standard. After the run, the gel was stained and then sliced. The pattern of gel staining and the distribution of radioactivity among the slices are shown in fig.2. Nearly all of the radioactivity was found coinciding with the E2 band whose position corresponds to app. M_r 74 000. Thus, E2 was not degraded by proteases. Since the same sample had shown M_r 52 000 in gel filtration, the anomalous behaviour of E2 is clearly established.

4. Discussion

A number of proteins have been reported to display

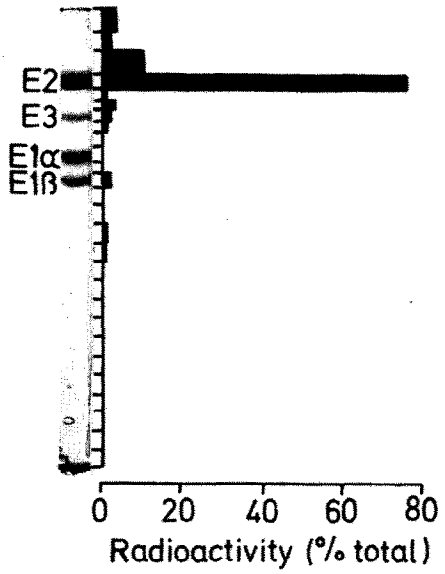

Fig.2. SDS–polyacrylamide gel electrophoresis of E2 after elution from the column. The stained bands represent, from top to bottom: E2 (M_r in SDS gel electrophoresis, 74 000); lipoamide dehydrogenase (E3) (M_r 56 000); pyruvate dehydrogenase (E1), α subunit (M_r 41 000) and β subunit (M_r 36 000). The gel was sliced as indicated on the left side of the scale and the radioactivity contained in the slices was counted. Total radioactivity (sum of all slices) was 5828 cpm.

anomalous migration in SDS gel electrophoresis (e.g. [15–17]). In contrast, anomalous behaviour of proteins in gel filtration with 6 M guanidine–HCl has not yet been reported [18]. Since, furthermore, M_r ~52 000 has now been found for the subunit of mammalian E2 by two independent methods, this value must be considered correct rather than M_r 74 000 resulting from SDS gel electrophoresis.

Two models have been proposed for the structure of the mammalian E2 core:

(i) A 60 subunit pentagonal dodecahedron with M_r 52 000 subunits [3];

(ii) A 24 subunit aggregate with subunits of M_r 74 000 [4,5].

The results reported here argue against the latter proposal. Furthermore, only a 60-subunit structure is in agreement with the symmetry of the E2 core as seen in electron micrographs [19,20].

M_r of the undissociated core has been determined to be ~3.1×10^6 ([3], bovine kidney and heart enzymes) or 1.8×10^6 ([4], pig heart enzyme). Since species differences between the bovine and porcine enzymes are very unlikely to cause such discrepancies, an obvious explanation suggested by our results would be that, in the latter study, E2 was fragmented by an endogenous protease to give a core consisting of M_r 60 000 ~ 30 000 subunits.

The reasons for the anomalous migration of kidney E2 in SDS gel electrophoresis may be similar to those discussed for *Escherichia coli* E2 [21]. This question is under study.

Acknowledgement

This work was supported by the Deutsche Forschungsgemeinschaft.

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